

Supplement Material

Reagents and antibodies. The following drugs and reagents were used freshly prepared or from stock solutions: nevirapine (100 μ M, 500 μ M, or 750 μ M in DMSO, Sigma, St. Louis, MO). The following reagents and antibodies were used for microscopy, Western blot, and co-IP studies described below: Paraformaldehyde (PFA 4% [2% final]), Alexa Fluor® 546 conjugate of wheat germ agglutinin (WGA, 1:1000 from Lifetechnologies, Eugene, OR), custom rabbit anti-L1 ORF1p antibody #984 (provided by Schumann GG¹), custom rabbit-anti-LINE-1 ORF2p (immunization peptide c+PKPGRDTTKKENFRP, 518-532, protein id AAD39215.1, Eurogentec, Liege, Belgium). This antibody was immuno-purified and verified for specificity by Western blot (quench experiment). A rabbit anti-LINE-1 ORF2p and goat anti-P-selectin (Santa Cruz, TX), goat anti-T7, mouse anti-myc-DDK, rabbit anti-LC3B and rabbit anti-ribosomal protein L26 (abcam, Cambridge, MA), mouse anti-DNA-RNA hybrid (Kerafast, Boston, MA), rabbit anti-TAP (Thermo Fisher Scientific, Waltham, MA), Click-IT AHA (L-Azidohomoalanine), and Click-IT AF488 alkyne (1mM, Lifetechnologies, Eugene, OR).

Platelet isolation and culture. All studies were approved by the institutional ethics committee (IRB#000392 and IRB#00077138). Platelets used for the described studies were freshly isolated from medication-free, healthy human subjects or, in select studies, from patients with HIV (see below). Platelets were leukocyte-reduced and isolated as previously described to yield a highly-purified population of cells with <1 leukocyte per 10⁵ platelets²⁴. Depleted platelets were resuspended at 1x10⁶/mL in serum-free M199 medium, placed in round-bottom polypropylene tubes (Becton Dickinson, Franklin Lakes, NJ), and cultured in a 37°C humidified incubator at 5% CO₂ for different time points. In select studies, platelets were treated with nevirapine or its vehicle control (DMSO).

RT-activity assays. RT-activity assays were performed as previously described⁵. In brief, platelets ($2 \times 10^7/200 \mu\text{l}$) or platelet derived RNPs (see below for details) were lysed in a buffer containing 10mM Tris-HCl pH 7.5, 1mM MgCl_2 , 1mM EGTA, 0.1mM PMSF, 5mM β -mercaptoethanol, 0.5% CHAPS, and 10% glycerol. In select experiments RNP lysates were immunodepleted for L1 ORF2p using the aforementioned anti-L1 ORF2p antibody and Agarose A/G plus beads (Lifetechnologies, Eugene, OR). The lysates were incubated on ice and finally centrifuged at 14,000 r.p.m. for 30 minutes to remove any cell debris. In select experiments the cell lysates were treated with nevirapine or its vehicle control (DMSO), or separated into retentate and filtrate using a 100kDa spin column (EMD Millipore, Billerica, MA, according to the manufacturer's protocol). RT activity was tested using the ThermoScript RT-PCR system (Lifetechnologies, Eugene, OR) in $20 \mu\text{l}$ reactions containing 10 ng of MS2 bacteriophage RNA (Roche Diagnostics, Basel, Swiss) or GFP-RNA (custom *in vitro* transcribed), which were DNase treated, RT-PCR buffer, 5mM DTT, 1mM of four nucleotide triphosphate mix, 5U of RNaseOUT, 15pmol of MS2 reverse primer and substituting commercial RT with cell-free extract (1.5×10^7 total cell amount equivalent in each reaction). After the RT reaction a $2 \mu\text{l}$ volume of the RT reaction were mixed with MS2 forward and reverse primers (5'-TCCTGCTCAACTTCCTGTCGAG-3', 5'-CACAGGTCAAACCTCCTAGGAATG-3') and PCR reaction was performed using the ThermoScript RT-PCR kit (Lifetechnologies, Eugene, OR). To exclude non-specific amplification reaction or contamination we included a panel of positive and negative control reactions as previously described⁵. *In vitro* assays of RT inhibition were performed in the same mixtures as used for the RT activity assay except that the extracts were pre-incubated with various doses of non-nucleoside reverse transcriptase inhibitors (NNRTI) prior to adding the MS2 RNA template.

RNA isolation. Platelets were lysed in TRIZOL, and RNA isolation was performed as previously described^{2,3}.

PCR and real-time qPCR studies. To determine LINE-1 mRNA expression pattern in human platelets, primers flanking ORF1 (5'-AGAAATGAGCAAAGCCTCCA-3' and 5'-GCCTGGTGGTGACAAAATCT-3') and ORF2 (5'-TCCAGCAGCACATCAAAAAG-3' and 5'-CCAGTTTTTGTCCCATTCAGT-3') were used (see schematic Figure 2D) and PCR was performed. PCR products were excised and subjected to Sanger-Sequencing using standard methods. MAP1LC3B mRNA expression was detected by PCR and qPCR (iCycler, BioRad, Hercules, CA) using the following primer set: 5'-GAAGTGGCTATCGCCAGAGT-3' and 5'-GATTTTGGTTGGATGCTGCT-3'. RNA was detected using the following primer set: 5'-ATGGACCTTGGTGAATTGTGTG-3' and 5'-TGGAAATGTGTCGTTTCCTATGG-3'.

Platelet *in situ* hybridization assays. For the assessment of *in situ* presence of LINE-1 mRNA and RNA in human platelets, platelets were fixed in suspension and spun down onto vectabond™ (Vector Laboratories, Burlingame, CA) coated coverslips as previously described⁶. *In situ* hybridization was performed using custom-designed and manufactured probes (see schematic Figure 2D) against LINE-1 according to the manufacturer's algorithms and protocols (Biosearch Technologies, Novato, CA).

Immunocytochemistry. Freshly isolated platelets were fixed in suspension, or placed in fibrinogen coated chamber slides to induce platelet spreading at various times (2, 6, or 18 hours), fixed in paraformaldehyde (PFA), and subsequently incubated with IgG or an antibody against LINE-1 ORF1p or ORF2p (see reagents and antibodies paragraph). In other experiments, platelets were either fixed immediately, to assess baseline RNA-DNA hybrid expression, or allowed to incubate for 18 hours in suspension. At the end of the experimental period, PFA was added directly to the platelets to maintain the native morphology of the cells, as previously

described^{3,4}. Fixed platelets were subsequently layered onto vectabond™ (Vector Laboratories, Burlingame, CA) coated coverslips using a cytospin centrifuge (Shandon Cytospin, Thermo Fisher Scientific, Waltham, MA). Platelets were permeabilized and RNA-DNA hybrids were specifically detected using the aforementioned antibodies. Specificity of the staining for anti-RNA-DNA hybrids was confirmed with isotype-matched non-immune IgG. To further confirm the specificity of the staining procedure, we used differential digestion of RNAs (RNase I, RNase A (high NaCl), RNase A (low NaCl), and Turbo DNase, all from Life Technologies, Eugene, OR).

Protein expression studies. All samples were normalized for starting cell concentrations. Platelets or platelet-derived RNPs were lysed in Laemmli-buffer, and samples were separated by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and examined by Western analysis for LINE-1 ORF1p, ORF2p expression pattern. Subsequently, proteins were detected by enhanced chemiluminescence (ECL). In quench experiments, the custom rabbit-anti-LINE-1 ORF2p antibody (see reagents and antibodies paragraph) was pre-incubated with its corresponding immunogenic peptide to demonstrate specificity. Additionally, P-selectin, ribosomal protein L26, and MAP1LC3B were detected using the same technique. Quantitation of Western blots was performed using ImageJ (NIH).

Flow cytometry. The expression of platelet surface adhesion molecules (e.g. PAC-1 binding to activated integrin $\alpha\text{IIb}\beta 3$) was evaluated by flow cytometry⁴. Freshly isolated platelets were cultured in the presence of Nevirapine 750 μM or its vehicle (DMSO, 6 hours), and subsequently activated with thrombin (0.1U/mL) for 10 minutes (Figure 4B). For Figure 4E, baseline platelets from healthy individuals and persons positive for HIV were evaluated. The cells were incubated with fluorescein isothiocyanate-conjugated antibody PAC-1 (no. 340535; BD Biosciences), fixed

and analyzed on a 5-color FACScan analyzer (BD Biosciences, USA) with appropriate isotype controls. Samples were analyzed using FlowJo v9 (Oregon, USA).

***In vivo* pulmonary embolism model.** All animal studies were approved by the University of Utah IACUC (protocol 15-10004). Eight to twelve-week old male and female mice C57Bl/6 mice were either administered 150 mg/kg nevirapine re-suspended in saline with 0.2% methylcellulose or vehicle control (saline with 0.2% methylcellulose) by oral gavage once a day for four consecutive days. On the fifth day, mice were subjected to a collagen/epinephrine pulmonary embolism model as previously described⁷. Cessation of breathing was monitored for 10 minutes before animals were euthanized by CO₂-asphyxiation.

Ribonucleoprotein particle (RNP) isolation. RNP isolation was performed according to Kulpa et al.⁸. In brief, platelets were lysed in RNP lysis buffer (1.5 mM KCl, 2.5 mM MgCl₂, 5 mM Tris-HCl [pH 7.4], 1% [w/v] deoxycholic acid, 1% [v/v] Triton X-100 and 1x Complete Mini EDTA-free protease inhibitor cocktail (Roche Diagnostics, Basel, Swiss)). Cleared supernatant was layered onto a sucrose cushion (8.5% and 17% [w/v] sucrose in 80mM NaCl, 5mM MgCl₂, 20mM Tris-HCl [pH 7.5], 1mM DTT, and 1x Complete Mini EDTA-free protease inhibitor cocktail). After a centrifugation step of 178,000 x g for 2 hr, the resulting pellet was lysed in TRIZOL for RNA isolation or in RT activity assay buffer to assess for RT activity.

***In vitro* translation of ORF1p and RNA binding and IP (RIP) studies.** We first *in vitro* translated ORF1p. In brief, a T7 promoter was put in front of the ORF1p coding segment of the pAD2TE plasmid (provided by J.V. Moran⁹) by using a PCR approach (T7 LINE-1 ORF1 forward 5'- TAATACGACTCACTATAGGGATGGGGAAAAACAGAACAG -3', T7 LINE-1 ORF1 reverse 5'- TTAACCCATTTGCTGTCCACCAGTCATGCTAGC -3'). The resulting PCR product was *in vitro* transcribed using the MEGAscript T7 kit, supplemented with the anti-

reverse cap analog (ARCA), and poly(A)-tailed using a poly(A)-tailing kit (all from Life Technologies, Eugene, OR). RNA was subsequently isolated and introduced into the 1-step human coupled IVT kit (ThermoFisher Scientific, Waltham, MA) according to the manufacturer's protocol. For all RIP studies the Magna RIP RNA-binding protein immunoprecipitation kit (EMD Millipore, Billerica, MA) was used according to the manufacturer's protocol. In brief, *in vitro* translated T7-tagged ORF1p was used in each reaction (specific antibody, and the isotype-matched non-immune IgG). The magnetic beads used for the IP reaction were prepared by incubating the beads with the antibody of interest (goat anti-T7, Abcam, Cambridge, MA) or the respective IgG control for 30 min. at room temperature (RT). IP was performed using the prepared beads, the supplied buffer, and the *in vitro* translated T7-tagged ORF1p with constant rotation overnight at 4°C. After several washes, total RNA (1.5 µg/sample) was isolated from platelets, added to the bead-bound protein, and incubated overnight at 4°C under constant rotation. Finally, beads were pelleted and RNA bound to ORF1p was isolated using TRIZOL according to the manufacturer's protocol. For cDNA synthesis, the superscript II RT-PCR system (Life technologies, Eugene, OR) was used. After the RT reaction a 2 µl volume of the RT reaction was mixed with ORF2 primer (5'-TCCAGCAGCACATCAAAAAG-3' and 5'-CCAGTTTTTGCCCATTCAGT-3'), or MAP1LC3B primer (5'-GAAGTGGCTATCGCCAGAGT-3' and 5'-GATTTTGGTTGGATGCTGCT-3'), and PCR reactions were performed.

Analysis of platelet morphology. For assessment of extended platelets with ≥ 2 cell bodies, platelets were isolated and resuspended at 1×10^6 /mL in serum-free M199 medium and cultured in a 37°C humidified incubator at 5% CO₂ for 6 hours in the presence of nevirapine or its vehicle (DMSO). The time course for these experiments was selected based on published studies from our lab identifying that 6 hours allows for extended cell body formation *ex vivo*⁴. Following the incubation period, cells were carefully fixed (PFA), spun on glass coverslips. Random fields (3

for each experimental condition) were recorded using microscopy techniques. Total platelets per field were counted (average 450 cells/field). Extended platelets were defined as platelets clearly possessing an extended morphology and ≥ 2 distinct cell bodies as previously described by our group and others^{4,10}. Changes in the formation of extended platelets were analyzed and compared to vehicle-treated conditions.

Protein *de novo* synthesis studies. Microscopy based global protein synthesis studies using Click-IT AHA (L-Azidohomoalanine) were performed as previously described⁴. For these studies, an average of 1,000 platelets per treatment arm were analyzed using CellProfiler software (see below for details). For global protein synthesis studies using radioactively labeled amino acids, purified platelets were resuspended at 1×10^6 /mL in serum-, methionine-, and cysteine-free DMEM medium (Lifetechnologies, Eugene, OR), placed in round-bottom polypropylene tubes (Becton Dickinson, Franklin Lakes, NJ), and cultured overnight in a 37°C humidified incubator at 5% CO₂ in the presence of [³⁵S]methionine and [³⁵S]cysteine. In select studies, platelets were treated with nevirapine or vehicle control (DMSO). Two-dimensional gel electrophoresis was performed as previously described⁴. In brief, platelets were lysed in buffer containing 7 M urea, 2 M thiourea, 4% CHAPS, 40 mM TRIS (base), and 2 tablets of a protease inhibitor mix per 20 ml of buffer stock solution (complete mini®, Roche Diagnostics, Basel, Swiss). Isoelectric focusing (IEF) was performed using the Protean IEF Cell (BioRad, Hercules, CA) at a temperature of 20°C. Gel strips (pH 3–10L, GE Healthcare, Marlborough, MA) were rehydrated for 12 h at 50 V using a buffer containing 7 M urea, 2 M thiourea, 2% CHAPS, 0.5% IPG buffer (pH 3–10L), DTT (2.8 mg/mL), and traces of bromophenol blue. The samples were applied as part of the rehydration solution and lysates were run on an 11 cm strip (linear gradient for 2 hours (100V), linear gradient for 1 hour (500V), linear gradient for 5 hours (1000V), linear gradient for 2.5 hours (8000V), and then a quick ramp and hold for 35 minutes (8000V). For the second dimension (SDS PAGE) IPG-strips were equilibrated for 20 minutes in buffer (6 M urea,

30% v/v glycerol (87% v/v), 2% w/v SDS, 50 mM Tris-Cl, pH 8.8, 100 mg DTT/10 mL, and traces of bromophenol blue). Gels (4-20%, Jule, Milford, CT) were coomassie stained using an optimized protocol from Neuhoff et al ^{11,12}, and subsequently dried and exposed to a Biomax MR film (Carestream, Rochester, NY) for autoradiographic detection.

RNA-DNA hybrid immunoprecipitation studies (modified RIP). For all modified RIP studies, the Magna RIP RNA-binding protein immunoprecipitation kit (EMD Millipore, Billerica, MA) was used according to the manufacturer's protocol. In brief, 24×10^9 total platelets were first lysed in 200 μ l of complete RIP lysis buffer. Next, 12×10^9 platelets were used in each reaction for the hybrid specific antibody, and the isotype-matched non-immune IgG. The magnetic beads used for the IP reaction were prepared by incubating the beads with the antibody of interest (anti-RNA-DNA hybrid) or the respective IgG control for 30 minutes at room temperature (RT). RIP was performed using the prepared beads, the supplied buffer and 100 μ l of the platelet cell lysate with constant rotation for 4 hours at 4°C. Phenol-chloroform isolation techniques were used for RNA purification with an additional overnight ethanol precipitation step. RNA yield was measured using the NanoDrop spectrometer. RNA-DNA hybrids were stored at -80°C until sequencing was performed.

Next generation RNA-sequencing. The cDNA libraries for deep sequencing were prepared using random hexamers. Samples were sequenced using the NEBNext Multiples Small RNA library Prep Set kit for 50 cycles as single reads, sequencing version 4, on an HiSeq sequencer and sequence reads were processed and aligned to hg38 as previously described ^{13, 14}.

Translational block experiments. The translational block experiments were based on the RT-activity assay described above. In brief, platelets ($2 \times 10^8/200\mu\text{l}$) were lysed in the RT buffer. The platelet lysate was pre-incubated with nevirapine ($500\mu\text{M}$ for 45 minutes at 37°C) with and without a melting step (2 minutes at 95°C) to separate RNA-DNA hybrids. The RT activity assay was set up in $20\mu\text{l}$ reactions containing 70.8ng of MAP1LC3B *in vitro* transcribed mRNA (True ORF RC207356, Origene, Rockville, MD; mMACHINE T7 Ultra Kit, Lifetechnologies, Eugene, OR), which was DNase treated. After the RT reaction, the resulting nucleic acids were introduced into an *in vitro* translation assay (1-step human coupled IVT kit, ThermoFisher Scientific, Waltham, MA). The resulting myc-DDK tagged MAP1LC3B protein was separated by SDS-polyacrylamide gel electrophoresis and examined by Western analysis for myc-DDK-tagged MAP1LC3B expression. Quantitation of Western blots was performed using ImageJ (NIH).

Studies in patients with HIV. All studies were approved by the institutional ethics committee (IRB#00077138). Platelets used for the described studies were freshly isolated as described above from consenting patients with HIV. Patients were excluded if taking immunosuppressant medications, had concurrent infection with hepatitis C, or another acute viral or bacterial infection, for which they were actively being treated or had been treated in the last 4 weeks.

Infection studies. DENV2 was propagated in C6/36 *Aedes albopictus* mosquito cells¹⁵ and titrated by plaque assay on LCC-MK2 cells. The amount of infectious particles was expressed as plaque forming units (PFU) per mL. Supernatants from uninfected cell cultures (mock) were produced using the same conditions. Freshly-isolated platelets (MOI 0.2) were infected with DENV2 and incubated for 24 hr with the virus before being analyzed.

Laser capture microscopy (LCM). Resting-state human platelets were isolated and fixed in suspension (see above). Platelets were spun down on a membrane slide (PEN membrane glass slide, Thermo Fisher Scientific, Waltham, MA). The samples were dehydrated using increasing ethanol concentrations and a final incubation step in Xylene for 5 min. For LCM we used a Veritas system (Arcturus Bioscience, Mountain View, CA). The laser power of the cutting laser was set to 2.28, the capture laser was set at 70 mA, pulse length 2000 μ sec, and a frequency of 10 hits. Membrane pieces were captured in macro LCM caps (Thermo Fisher Scientific, Waltham, MA). RNA from target cells was isolated using the PicoPure RNA isolation kit (Thermo Fisher Scientific, Waltham, MA), and subsequently amplified using the RiboAmp HS PLUS Kit (Thermo Fisher Scientific, Waltham, MA). cDNA synthesis and PCR reactions for LINE-1 ORF1 and ORF2 were performed as described above.

Microscopy and image analysis. Fluorescence microscopy and high resolution confocal reflection microscopy was performed using an Olympus IX81, FV300 (Olympus, Melville, NY) confocal-scanning microscope equipped with a 60x/1.42 NA oil objective for viewing platelets. An Olympus FVS-PSU/IX2-UCB camera and scanning unit and Olympus Fluoview FV 300 image acquisition software version 5.0 were used for recording. In addition, an EVOS FL Auto Cell imaging system with integrated dual camera system, system specific software and equipped with a 60x/1.42 NA oil objective was used. Monochrome 16 bit images were further analyzed and changes quantified using Adobe Photoshop CS6, ImageJ (NIH), and CellProfiler (www.cellprofiler.org)^{16,17}.

Statistical analyses. The mean \pm SEM was determined for each variable. Student two tailed t-tests or ANOVA was used to identify differences among two or multiple experimental groups respectively. If significant differences were found, a Newman-Keuls post-hoc procedure was used to determine the location of the difference. A two-tailed p-value <0.05 was considered significant.

Supplemental References

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